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Environmental Contamination and Toxicology

# Interaction of Chlorinated Phenolics and Quinones with the Mitochondrial Respiration: A Comparison of the o- and p-Chlorinated Quinones and Hydroquinones

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Interest in the environmental toxicology of chlorinated catechols and their analogous quinones was prompted by their acute toxicity towards fish and other aquatic organisms (Das et al. 1969; Leach and Thakore,, 1975). These chlorinated substances are by-products of chlorination reactions and have been identified in the discharges from pulp and paper mills (Leach and Thakore, 1975; Lindstrom and Nordin, 1976) making them potentially important environmental pollutants.

Chlorophenols, such as pentachlorophenol, as well as tetrachlorocatechol have been suggested to uncouple mitochondrial oxidative phosphorylation (Arrhenius et al. 1977; Lundberg et al. 1980; Weinbach, 1954) while chloranil and tetrachloro-o-benzoquinone have been shown to inhibit liver mitochondrial respiration (Lundberg et al. 1980), which may be related to their cytotoxicity. Another chlorinated quinone fungicide, 2,3 dichloro-1,4-naphthoquinone (CNQ) has been studied and shown to both uncouple oxidative phosphorylation and inhibit respiration in liver and heart mitochondria (Pritsos et al. 1982). CNQ was shown to undergo redox cycling with mitochondria, with a concomitant production of toxic oxygen species including superoxide and hydrogen peroxide. These reactive oxygen species were associated with the generation of mitochondrial oxidative stress, and were related to the toxic action of CNQ (Pritsos and Pardini, 1984). Based upon these previous findings, we examined the interaction of both the ortho and para isomers of tetrachloro-benzoquinone and their corresponding hydroquinones with mitochondria in order to probe their mechanism of actions and compare the reactions of the various isomers.

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## MATERIALS AND METHODS

Tetrachloro-p-benzoquinone (chloranil) and tetrachlorohydroquinone were obtained from Sigma Chemical Co. (St. Louis, MO). Tetrachloro-o-benzoquinone was obtained from Aldrich Chemical Co. (Milwaukee, WI) and tetrachloropyrocatechol (tetrachloro-o-hydroquinone) was obtained from Pfaltz and Bauer Inc. (Waterbury, CT). All other materials were obtained from standard commercial sources.

Heavy beef heart mitochondria (HBHM) were isolated by differential centrifugation and stored as previously described (Smith, 1967). The mitochondria were aged by repeated freezing and thawing so that respiration was uncoupled from phosphorylation. The HBHM succinoxidase enzyme activity was determined manometrically in the absence and presence of the test compounds at 30°C as previously described (Cheng and Pardini, 1979). The total volume of respiration buffer was 2.8 ml and contained 0.5-1.0 mg protein. The buffer was at pH 7.4 and it contained 33 mM Tris, pH 7.4, 166 mM sucrose, 0.12 mg cytochrome c (Sigma grade III) and 0.06 ml of asolectin, a soybean phospholipid in micellar form (Associated Concentrations, Woodside, New York) prepared as previously described (Cheng and Pardini, 1979). The quinones were added in ethanol or ethanol:DMSO (1/1) according to their solubilities, which was maintained constant at 2.8% v/v in all of the reaction vessels. Succinate was added in 0.2 ml water to a final concentration of 150 uM. The reaction was initiated following a 10 minute thermal equilibration by tipping the substrate from the side arm into the main compartment. KOH and a paper wick placed in the center well trapped the evolved $CO_2$. Oxygen volume was recorded at 5 minute intervals and only those assays giving a linear rate of $O_2$ consumption for a minimum of 15 minutes were included in the assay. In those experiments requiring continual monitoring of HBHM oxygen consumption, it was accomplished polarographically employing a YSI oxygen meter equipped with a Clark electrode (Estabrook, 1967).

## RESULTS AND DISCUSSION

HBHM succinoxidase activity was monitored manometrically in the presence and absence of the four compounds tested (Figures 1 and 2). These data show that tetrachloro-p-hydroquinone is the most potent succinoxidase inhibitor of the four compounds tested with tetrachloro-p-benzoquinone next, followed by tetrachloro-o-hydroquinone and tetrachloro-o-benzoquinone with $I_{50}$ values of 5,18,20 and 44 nmoles/mg protein respectively. These $I_{50}$ values were obtained by extrapolation from the inhibition titration curves.

The four compounds were next tested for their ability to autoxidize in a buffered solution by continually measuring oxygen consumption with a Clark electrode. The data in Table 1 show that only tetrachloro-p-hydroquinone exhibited measurable oxygen consumption under these conditions. In order to determine if the various test chemicals were capable of donating electrons to the

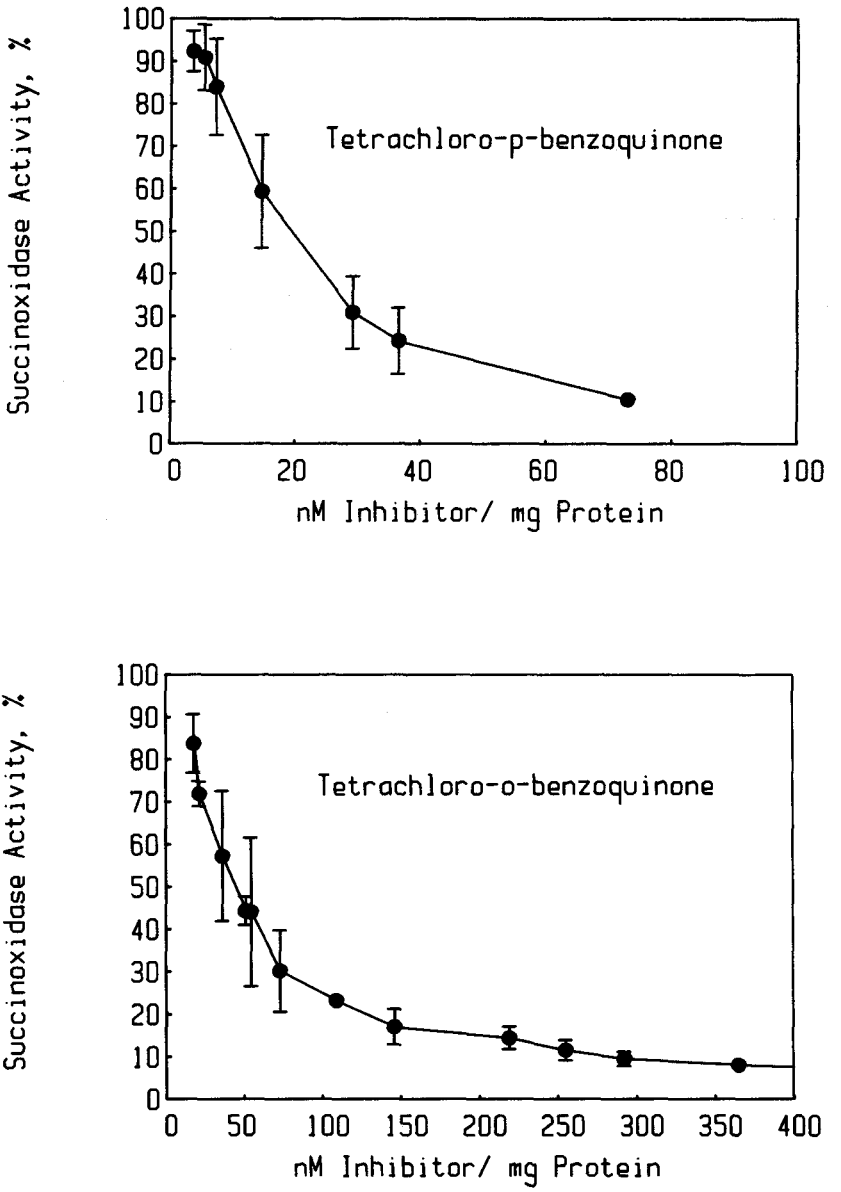
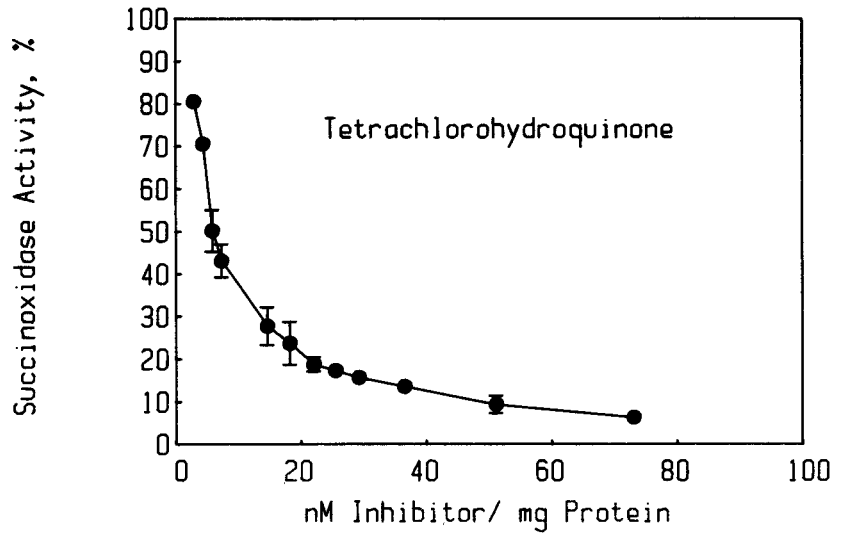



Figure 1 Inhibition of mitochondrial succinoxidase respiration by chlorinated benzoquinones. Assays were run as previously described in the Materials and Method section. Each value represents an average of at lease three determinations ± standard deviation.



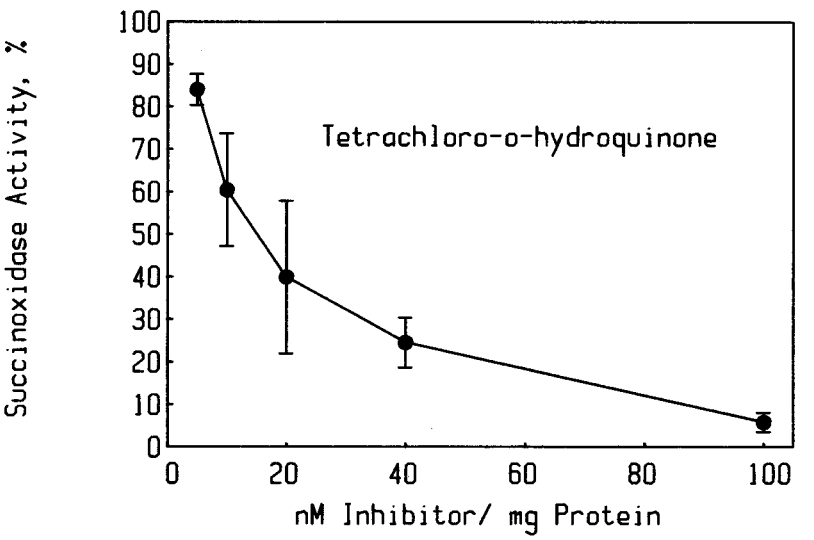




Figure 2 Inhibition of mitochondrial succinoxidase respiration by chlorinated hydroquinones. Assays were run as previously described in the Materials and Method section. Each value represents an average of at lease three determinations ± standard deviation.

mitochondrial respiratory chain, we measured mitochondrial oxygen consumption in the absence of substrate and in the absence and presence of cyanide, a cytochrome oxidase inhibitor. The addition of tetrachloro-o-hydroquinone and tetrachloro-p-hydroquinone to HBHM resulted in a greater rate of oxygen consumption than that observed in the absence of mitochondria. Conversely, the addition of tetrachloro-p-benzoquinone and tetrachloro-o-benzoquinone to HBHM in the absence of respiratory substrate did not stimulate oxygen consumption, Table 1. The addition of cyanide during tetrachloro-p-hydroquinone or tetrachloro-o-hydroquinone induced HBHM oxygen consumption depressed the rate of oxygen consumption of the two compounds to a rate similar to their rate of auto-oxidation, Table 1. This suggests that the enhanced oxygen consumption of these two compounds is due to the donation of reducing equivalents to the HBHM respiratory chain, resulting in terminal electron transport through cytochrome oxidase.

Quinones have been shown to undergo redox cycling in the presence of mitochondria and microsomes when a source of reducing equivalents is available (Doroshow and Davies, 1986; Pritsos and Pardini, 1984). We tested the relative abilities of each of these four compounds to redox cycle with the succinoxidase component of the HBHM respiratory chain. Mitochondrial respiration was initiated by the addition of succinate which was then inhibited by cyanide. Drug was subsequently added and oxygen consumption monitored. Cyanide insensitive respiration in mitochondria is indicative of quinone redox cycling (Pritsos and Pardini, 1984). A representative tracing is shown in Figure 2 for tetrachloro-p-benzoquinone. The data obtained from these experiments showed that both tetrachloro-p-benzoquinone and tetrachloro-p-hydroquinone were capable of redox cycling with the succinoxidase enzyme system. Tetrachloro-p-hydroquinone showed much greater cyanide insensitive oxygen consumption than tetrachloro-p-benzoquinone; however subtraction of the rate of autooxidation in the presence of mitochondria and cyanide as shown in Table 1 indicated that tetrachloro-p-benzoquinone was actually better at redox cycling with succinoxidase. Neither of the ortho compounds were capable of redox cycling in this system although tetrachloro-o-hydroquinone consumed oxygen at a rate equivalent to its rate of autooxidation in the presence of mitochondria. The addition of catalase during the redox cycling experiment, as shown in Figure 2, resulted in the initial regeneration of oxygen and a subsequent slowing of the oxygen consumption rate. This evidence indicates that hydrogen peroxide is being formed during the redox cycling reaction (presumably via superoxide dismutation) as catalase converts $H_2O_2$ to water and molecular oxygen. The addition of superoxide dismutase prior to drug addition (to more efficiently convert superoxide to hydrogen peroxide) resulted in a greater catalase effect, implicating superoxide as a precursor of hydrogen peroxide (data not shown). These findings demonstrate that tetrachloro-p-hydroquinone and tetrachloro-p-benzoquinone are both capable of redox cycling with the mitochondrial succinoxidase enzyme system, resulting in the generation of superoxide and hydrogen peroxide into the surrounding media.

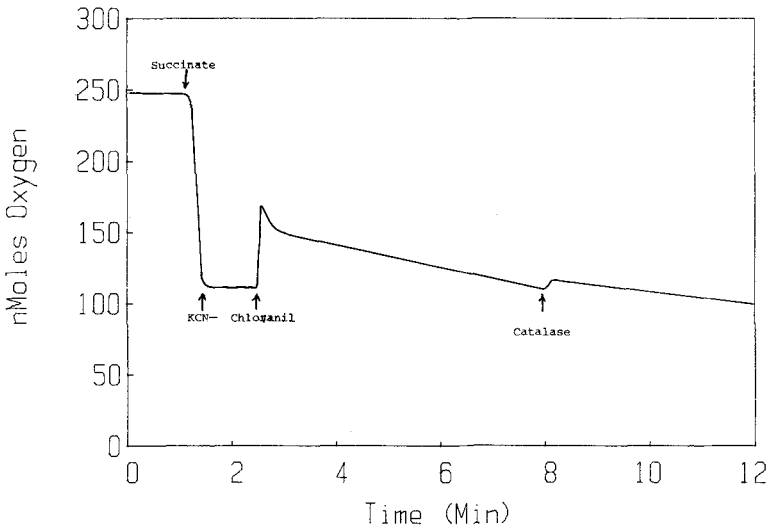



**Figure 3** Chloranil induced cyanide insensitive mitochondrial respiration. One mg HBHM in two ml of 33 mM Tris buffer with asolectin (pH 7.4) were treated with succinate (3.3 mM final conc.) and subsequently HBHM succinoxidase respiration was inhibited by 1mM KCN . Chloranil, 1.25mM final conc. (dissolved in a 50:50 mixture of DMSO:ethanol) was added and oxygen consumption was monitored for several minutes. The addition of 0.1 mg catalase was made during a time period previously established to be linear for oxygen consumption under these experimental conditions. The increase in oxygen at the point of chloranil addition is due to dissolved oxygen in the ethanol solvent. This is a representative tracing obtained from several assays.

Table 1 Autooxidation of Chlorinated Benzoquinones

| Compound (1.25 mM) | Oxygen Consumption (nMoles $O_2$/min) | Oxygen Consumption in the presence of mitochondria (nMoles $O_2$/min) | Oxygen Consumption in the presence of mitochondria +KCN (nMoles $O_2$/min) |
|---|---|---|---|
| Tetrachloro-o-Benzoquinone | 0 | 0 | 0 |
| Tetrachloro-o-Hydroquinone | 0 | 9.9 | 0 |
| Tetrachloro-p-Benzoquinone | 0 | 0 | 0 |
| Tetrachloro-p-Hydroquinone | 36 | 163 | 45 |

Four compounds were monitored for their ability to induce oxygen consumption in the presence of buffer (33mM Tris buffer with asolectin pH 7.4), buffer + 1 mg HBHM and buffer, 1 mg HBHM and 1mM KCN . Each value represents an average value obtained from at least 5 determinations.

The data from these experiments suggest that for this series of compounds, the para quinone configuration is more inhibitory to succinoxidase than the ortho quinone. It also appears that the ortho and para hydroquinones are better inhibitors than their respective quinone counterparts. The para quinone and hydroquinone configurations underwent redox cycling and generated oxygen radicals whereas the ortho analogs did not. The ability to generate these toxic oxygen species may account for the more potent inhibition by the para compounds. The rapid autooxidation of tetrachloro-p-hydroquinone, which generates oxygen radicals, may account for its enhanced toxicity to HBHM over tetrachloro-p-benzoquinone. It has been previously demonstrated that oxygen radicals are capable of causing mitochondrial damage resulting in mitochondrial swelling, depletion of sulfhydryl groups and lipid peroxidation (Pritsos et al. 1982; Pritsos and Pardini, 1984). These data therefore suggest that there may be two or more components to the chlorinated benzoquinone toxicity to mitochondria: 1) a direct quinone interaction with the mitochondria and, 2) an oxygen radical component. In addition, the observation that the two hydroquinone compounds donate electrons to the respiratory chain may be a third important component of their toxic action. For example, if this electron donation were a 1 electron process, then a semi-quinone would be generated which in turn could react with molecular oxygen to generate oxygen radicals or the semi-quione may further interact with redox carriers in the mitochondrial respiratory chain. Either of these events could lead to mitochondrial inhibition.

Environmentally, tetrachloro-p-hydroquinone probably rapidly autooxidizes to chloranil, which is very toxic. Tetrachloro-o-hydroquinone, which appears to be almost equitoxic to mitochondria as tetrachloro-p-benzoquinone, does not autooxidize rapidly and these compounds could therefore be a concern in the environment if generated in sufficient quantities and released through the discharges from pulp and paper mills into nearby waterways.

Although these findings report than the chlorinated phenolic and quinoid compounds interact with the mitochondrial respiratory chain *in vitro*, the role of the mitochondrial interaction in the toxicology of these compounds *in vivo* remains to be established. A structure-activity study with 12 structurally related naphthoquinones revealed that redox potential, inhibition of HBHM succinoxidase and cytotoxicity were closely related (Hodnett et al. 1983; Pisani et al. 1984; Pisani et al. 1986). Saxena et al. (1974) demonstrated that treatment of L mouse fibroblasts with the chlorinated naphthoquinone fungicide, CNQ, which interacts with mitochondrial succinoxidase to generate oxygen radicals (Pritsos et al. 1982) resulted in the formation of large protruding blebs on the cellular membrane and irreversible swelling of the cells. By analogy, these findings suggest that the succinoxidase inhibitors reported herein will also possess *in vivo* activity.

REFERENCES

Arrhenius E, Renberg L, Johansson J, Zetterqvist M.-A, (1977) Disturbance of microsomal detoxication mechanism in liver by chlorophenol pesticides. Chem -Biol Interact 18:35-46.

Cheng SC, Pardini RS (1979) Inhibition of mitochondrial respiration by model phenolic compounds. Biochem Pharmacol 28:1661-1667.

Das BS, Reid SG, Betts JL, Patric K (1969) Tetrachloro-o-benzoquinone as a component in bleached craft chlorination effluent toxic to young salmon. J. Fish Res. Bd. Can. 26:3055-3059.

Doroshow JH, Davies KJA (1986) Redox cycling of anthracyclines by cardiac mitochondria. J. Biol. Chem. 261:3068-3074.

Estabrook RW (1967) Mitochondrial respiratory control and the polorigraphic measurement of ADP:O rations. In: Estabrook RW, Pullman ME (eds) Methods in Enzymology, Academic Press, New York, 10:41-47.

Hodnett EM, Wongwiechintana C, Dunn III WJ, and Marrs PJ (1983) Substituted, 1,4-Naphthoquinones vs the Ascitic Sarcoma 18° of Mice. J. Med. Chem. 26:570-574.

Leach JM, Thakore AH (1975) Isolation and identification of constituents toxic to juvenile rainbow trout (Salmon gairdneri) in caustic extraction effluents from craft pulpmill bleach plants. J. Fish Res. Bd. Can., 32:1249-1254.

Lindstrom K, Nordin J (1976) Gas chromatography-mass spectrometry of chlorophenols in spent bleach liquors. J. Chromatogr. 128:13-18.

Lundberg L, Renberg L, Arrhenius E, Lundstrom G, (1980) Detoxication disturbances and uncoupling effects in vitro of some chlorinated guaiacols, catechols and benzoquinones. Chem.-Biol. Interactions, 32:281-290.

Pisani DE, Elliott AJ, Hinman DR, Pardini RS (1984) Relationship between inhibition of mitochondrial respiration by naphthoquinones to anti-tumor activity and redox potential. Fed. Proc. 43:1543.

Pisani DE, Elliott AJ, Hinman DR, Aaronson LM, Pardini RS (1986) Relationship between inhibition of mitochondrial respiration by naphthoquinones, their antitumor activity and their redox potential. Biochem. Pharmacol. 35:3791-3798.

Pritsos CA, Jensen DE, Pisani D, Pardini RS, (1982) Involvement of superoxide in the interaction of 2,3-dichloro-1,4-naphthoquinone with mitochondrial membranes. Arch. Biochem. Biophys. 217:98-109.

Pritsos CA, Pardini RS, (1984) A redox cycling mechanism of action for 2,3-dichloro-1,4-naphthoquinone with mitochondrial membranes and the role of sulfhydryl groups. Biochem. Pharmacol. 33:3771-3777.

Saxena J, Sikka HC, Zweig G (1974) Structural and functional changes in mouse fibroblast cells treated with 2,3-dichloro-1,4-naphthoquinone (dichlone). Pest. Biochem. Physiol. 4:185-189.

Smith AL (1967) Preparation, properties and conditions for assay of mitochondria: Slaughterhouse material small scale. In:

Estabrook RW, Pullman ME (eds) Methods in Enzymology, Academic Press, New York, 10:81-86.
Weinbach EC (1954) The effect of pentachlorophenol on oxidative phosphorylation. J. Biol. Chem., 210:545-550.

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